A MORPHOLOGICAL STUDY OF ULMUS AMERICANA. CONTRIBUTIONS FROM THE HULL BOTANICAL LABORATORY. LXXVIII.

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(WITH PLATES VII-IX)

A STUDY of this species was suggested by the interesting results of recent investigations in this region of the Archichlamydeae; notably those of Karsten (12), Miss Benson (1), Zinger (22), and Nawas-Chin (16).

METHODS.

Collections were made from February 13 to May 1, 1903, and repeated during the same period for 1904. During the first year collections were made on alternate days throughout the more rapid period of growth, and at intervals of ten days at other times. During the second year the same plan was followed except that collections were made every day during the period of fertilization and embryo growth.

The ovules are covered with a dense growth of hair which prevents sinking in the killing fluid; but after immersion in 95 per cent. alcohol they sink at once. A 2 per cent. solution of chromo-acetic acid was found to give the best results as a killing agent for all but the oldest stages; these requiring a somewhat stronger solution.

The material was imbedded in paraffin and the sections were cut from 2 to 10 \mu in thickness. A preparation of Le Page's glue and glycerin was used for fixing sections to the slide (glue 40 parts, water 10 parts, glycerin 50 parts). The albumen and several other fixatives were first tried, but all failed to fix the sections to the slide. The glue mixture is as perfectly transparent on the slide as Mayer's albumen fixative, is more easily prepared, is a much stronger adhesive, will keep indefinitely, and is not so easily coagulated by heat.

The most satisfactory combination for staining the ovules was found to be safranin and gentian violet. The addition of orange G to the above brought out the pollen tubes best, as they hold the gen-

tian violet after it is drawn from the nucellus and integuments. The male cells stained best in orange G. Haidenhain's iron-haematoxylin gave good results, as did also gentian violet.

FLOWERS.

The earliest stages in the development of the flower were not studied. The first collecting was done on February 13, when the ovule was found to contain a clearly defined megaspore, and the anthers to be in the pollen mother cell stage. By March 25 the trees were in full bloom.

The method by which self-pollination is prevented, at least to a large extent, is of interest. When the flower bud first opens, the two-parted stigma is found protruding beyond the anthers and is ready for pollination (fig. 1). About two of the more centrally placed flowers in each cluster are somewhat earlier than the others in lengthening their flower stalks and filaments and in opening their anthers (fig. 2). The first flower to open its anthers has an excellent opportunity to pollinate the entire cluster. At the same time this flower may be prevented from self-pollination only by the pollen from some earlier flower having reached its exposed stigma before its own anthers were opened. In many instances the stigmas of flowers whose anthers were not yet open were found covered with pollen grains, some of which had developed tubes. As the time requi ed for the pollen tube to complete its growth is from one to three days, it is quite evident that these early tubes will have completed the act of fertilization in each flower before its own pollen grains have an opportunity to begin the development of tubes.

MICROSPORANGIUM.

On February 13 the microsporangia were well formed. Most of the sporogenous cells of the four chambers were in the spore mother cell stage, in which they had evidently passed the winter (fig. 3).

It is of interest to note that at this stage it is impossible to distinguish any definitely organized tapetum, the cells all having the same size and shape, and giving the same reaction to stains. Sporangia of the same date were found in which the mother cells had passed into the synapsis stage (fig. 4); the nucleolus staining red and the chromatin mass violet. Many of the cells which were functioning

as tapetum contained two or three nuclei and abundance of food material which stains deeply.

The tapetum consists generally of a single layer; and is derived from the original sporogenous mass. This is clearly shown by the fact that the two layers of cells within the endothecium never contribute to the formation of tapetum, but break down early while the endothecium itself enlarges (fig. 5). That the tapetum is derived from the original sporogenous mass is further shown by its extension inward, sometimes to the depth of several layers (fig. 4), more or less intermingled with the cells which are functioning as spore-forming tissue. Bower (2) has shown that in Equisetum from one-fourth to one-third of the sporogenous cells disorganize and do not form spores. He says "their function is that of a diffused tapetum and there can be no doubt that their substance contributes to the nutrition of the survivors." This contribution seems to be very evident in Ulmus.

Wylie (21) has shown in Elodea that there is a regular contribution to the tapetum from the sporogenous mass; Webb (20) shows in Astilbe that the tapetum has the same origin as the spore mother cells; Land (14) notes that in Ephedra it is often impossible to distinguish the tapetal cells from adjacent mother cells, mentioning that the tapetum seems to be potentially sporogenous tissue which has become sterile by virtue of its position; and Coulter (6) states that in Ranunculus it seemed as if the whole tapetum were cut off from the periphery of the sporogenous mass. The tapetum in Ulmus is surely composed of sterilized sporogenous cells.

On February 15 the pollen mother cells are just beginning to pass from their winter stage, for on the same slide mother cells were found in the resting stage (fig. 3), in synapsis (fig. 4), in the first mitosis (fig. 6), and in the second mitosis (fig. 7). Ten days later the tetrads were well formed (fig. 5); also there were many cells containing four free nuclei, evidently just preparing to form microspores (fig. 8).

The tapetum begins to break down about March 1, and by March 12 is entirely absorbed. At this time the tetrads are uninucleate (fig. 9). The two inner layers of the sporangium wall also rapidly break down and disappear. At the same time the endothecium enlarges; its cells take on a cork-like appearance and do not stain

well; later the irregular thickening bands appear in its cells (fig. 5).

The filaments do not begin to elongate until March 20, when they extend quite rapidly, the microsporangium being fully mature March 26.

MEGASPORANGIUM.

On February 13 the megasporangium consisted of the nucellus containing a single hypodermal archesporial cell and a single integument. The archesporial cell divides by a periclinal wall, and the outer daughter cell also divides by a periclinal wall, giving rise to two parietal layers of cells (fig. 23); one of these occasionally again divides. The megasporangium evidently passes the winter in the megaspore mother cell stage, thus being identical in this particular with the microsporangium.

By February 15 the mother cell begins to enlarge. It accomplishes this chiefly by elongation, the long embryo sac pushing its somewhat pointed lower extremity deep into the tissue of the nucellus (fig. 24). The second integument appears February 25. By March 15 the first integument has closed over the top of the nucellus (fig. 27), whose crown cells have already begun to enlarge and divide preparatory to forming the long beak-like or archegonium-like necks shown in figures of more mature stages. These archegonium-like necks strongly resemble those figured by Miss Lyon (15) for Euphorbia corollata. They differ however from those of Euphorbia in that they do not project through or beyond the integuments, but press against them. Possibly the rapid anticlinal divisions of the cells of the inner integument cause the elevation, thus forming a dome-like cavity into which the beak-like tip of the nucellus grows (figs. 27-35). The integuments are fully developed by March 25. A third integument was clearly made out in a number of instances (fig. 27), which is probably due to the splitting of the outer integument.

MALE GAMETOPHYTE.

The mother cells were found in the first and second mitosis on February 15 (figs. 6-7). About February 25 there appears a distinct though delicate wall about each of the four young spores, which are still enclosed by the wall of the mother cell (fig. 9). The wall

of the mother-cell gradually breaks down and by March I the microspores are rounding off; many of them have formed their tube and generative nuclei. These are at first very much alike, but the tube nucleus soon becomes larger and stains more deeply (fig. 10). At this time the two coats can be clearly distinguished, the exine having acquired an uneven, reticulate surface and showing five very distinct openings (figs. 10-11) through which the intine can be seen.

The division of the generative nucleus was observed March 23, or before the dehiscence of the sporangia (fig. 12). At this time the tube nucleus shows signs of disintegration which is completed by March 26. The tube nucleus was often found disintegrating when the pollen tube was just starting, and was never found to leave the pollen grain, and in my judgment it never does so in Ulmus americana. Perhaps this is due to the fact that the pollen tube is not

more than 3^{mm} in length.

It was definitely determined that the male structures are cells, and not merely nuclei, the delicate limiting membranes being clearly made out. During a large part of their existence in the pollen grain these lenticular cells are attached to each other by their adjoining ends in such a manner as to make them appear in longitudinal section as if astride of the tube nucleus (fig. 13). WYLIE (21) has shown that in Elodea the male cells are attached in a similar manner.

FEMALE GAMETOPHYTE.

The mother cell does not form the usual tetrad, but functions directly as a megaspore (fig. 23). This condition is well known in many angiosperms. While no instance of more than one megaspore was found, the fact that there are frequently two embryo sacs in the older stages at once suggests the possibility that the megaspore mother cell in Ulmus may yet be found like that of Juglans, a closely allied form, to vary in the number of megaspores it forms, or possibly to form occasionally two mother cells. This would account for the double embryo sacs (figs. 55-56).

In the early part of February the megaspore shows only slight differentiation, being but little larger than the adjacent cells (fig. 23). The nucleus however is quite large, deeply staining, and begins to

show signs of preparation for division.

The actual mitosis resulting in the binucleate embryo sac was not observed, but binucleate sacs were found March 11 (fig. 25), in which the spindle fibers between the nuclei had not yet disappeared. By March 16 these nuclei had again divided, showing a great variation in the arrangement of the resulting four nuclei (figs. 26, 27, 60). On March 17 the third mitosis (fig. 28) shows one nucleus dividing parallel to the main axis and three at right angles to it. Figs. 29-31 are even more perplexing than the foregoing, showing that rapid divisions have occurred in various planes.

After reaching the eight-nucleate stage there are, in a majority of cases, no further nuclear divisions; the egg apparatus begins to organize, the antipodals take their proper place, and the polar nuclei move toward each other preparatory to fusion (fig. 30). However, in very many cases, there is further nuclear division without any indication of polarity, the nuclei being distributed promiscuously throughout the cytoplasm of the sac and all apparently alike (figs.

31-55).

Mitotic figures were not found in the sac after the eight-nucleate stage was reached, but many sacs were examined containing as high as twelve (occasionally more) free nuclei very evenly distributed and very similar in appearance. Later a number of embryo sacs were found having more than eight nuclei and showing polarity. In these four nuclei were in the micropylar and eight or more in the antipodal end of the sac (fig. 32). Fig. 54 shows the only observed exception to the above rule. (These numbers include also the nuclei which are to function as polars at a later date.)

The antipodals, excepting two or three, soon disintegrate. The remaining ones enlarge rapidly, sometimes rivaling the egg in their prominence (figs. 53-54). They seem, however, to be of the passive

type common among Archichlamydeae.

The embryo sac of Ulmus americana, therefore, shows a condition intermediate between the regular eight-nucleate angiosperm type and the sixteen-nucleate sac of the Peperomia described by CAMPBELL (3) and JOHNSON (II).

The fusion of the eight nuclei to form the endosperm nucleus in Peperomia has its parallel in the fusion of several nuclei in Ulmus for the same purpose. NAWASCHIN (16) has reported an instance of three polar nuclei fusing in Ulmus and I have frequently observed three or four nuclei in contact and evidently preparing to fuse (figs. 58-59). Several cases were found where a well formed egg appeared in the antipodal end of the sac (figs. 36, 50, 54, 56). Notwithstanding the fact that in each of these cases the structures seemed to be normal eggs in every particular, I hesitated to adopt this interpretation until later, when embryos were discovered in the antipodal ends of two sacs, and in each of which a larger and older embryo appeared in the micropylar end (figs. 51-52). These antipodal embryos are wholly within the sac and I do not think they were produced apogamously.

In 1895 Chamberlain (5) found in Aster novae-angliae what he termed an antipodal oosphere, calling attention to its cytological resemblances to the ordinary oosphere, and stating that "we need only fertilization and the formation of an embryo to completely establish its right to the name."

Lately Miss Oppermann (17) has found an antipodal egg in Aster undulatus, with the sperm lying against the cytoplasm of the

egg, thus proving that fertilization does actually occur.

Tretjakow (19) has found the antipodal embryo which, since Miss Oppermann's discovery we are justified in concluding comes from a fertilized antipodal egg, thus making the history complete and establishing beyond a reasonable doubt the right to call this structure an egg. As the conditions mentioned above were all found in Ulmus, I feel justified in calling these antipodal structures eggs. There are two well formed synergids which enlarge nearly to the size of the egg. One of these usually disappears about the time the pollen tube enters the sac. The other generally persists until after the first division of the egg.

The polar nuclei were never found actually fusing, though they were often found in close contact (fig. 33), in which condition they

seem to remain for some time.

Wylie (21) has shown that in Elodea the actual presence of the pollen tube in the ovule is necessary in order to stimulate fusion. Guignard (10) has arrived at the same conclusion in regard to Capsella. I find that the polar nuclei of *Ulmus americana* behave in the same manner, fusion occurring at least before fertilization.

FERTILIZATION.

The most interesting feature in connection with fertilization is the behavior of the pollen tubes. These begin to project through the openings in the exine about March 26, usually presenting a single tube for each pollen grain (fig. 15). While this is the general rule, it is by no means always the case, as many pollen grains were found developing from two to five tubes (figs. 18–19). In fig. 18 all the tubes seem to have had an equal stimulus to growth, but such is not the case in fig. 19. In this instance the largest tube was in contact with the stigmatic hair, which fact doubtless accounted for its greater size. Eventually one of these tubes gains the ascendency over the others which are drawn back into the microspore as it gradually shrivels and the tube elongates (fig. 17). This figure also shows the peculiar method of the young tube on coming in contact with the stigmatic hair, down which it almost invariably travels to reach the stigma.

It was noted frequently that the tube when meeting the hair nearly at right angles would direct its course towards the distal end instead of towards the stigmatic end, as might be expected. After reaching the end of the hair the tube would often form a cyst-like enlargement before proceeding downward to the stigmatic tissue.

The behavior of the pollen tubes within the tissue has been so accurately described by Nawaschin (16) for *Ulmus montana* and *Ulmus pedunculata* that it will not be necessary to dwell upon their behavior in *Ulmus americana*. Suffice it to say, the same branching and apparently aimless wandering through the funiculus, integuments, and occasionally the nucellus which he describes was noted. In a few cases these tubes were found anastomosing about the micropylar end, as shown in *fig. 22*. The tube, after pre sing its way through the micropyle, enters the nucellus near the tip of the beak (*fig. 35*) and passes directly to the upper end of the embryo sac. The only cases where I observed branching were those of belated tubes entering the ovule after fertilization (*figs. 21–22*). Such tubes seem to have a general tendency to push toward the antipodal end of the sac. In fact there is some indication that they occasionally reach the chalaza.

The pollen tube is not easily disintegrated after fertilization, and is found intact, though staining feebly, until the embryo has from

sixteen to twenty-four cells. Two tubes passing down the same micropyle were occasionally noted (fig. 20).

The male cells lose their cytoplasm on entering the pollen tube, and during their journey to the embryo sac are simply elongated nuclei (figs. 16, 33). They were found side by side in the tube soon after leaving the pollen grain and were still somewhat elongated and very close together on entering the embryo sac, where the tube enlarges in a very irregular cyst-like manner (fig. 33). After entering the sac the nuclei become spherical and begin to gather a small amount of cytoplasm around them. The first to enter the sac generally fuses with the fused polars (fig. 34), the second fusing with the egg. Fertilization occurs from March 29 to April 1.

ENDOSPERM.

The endosperm begins to form soon after the male nucleus fuses with the fusion nucleus (fig. 34). This almost always occurs before fertilization, but instances were noted where fertilization probably occurs first (fig. 37). This variation was noted by LAND (13) in Erigeron, where he found sometimes the egg and at other times the endosperm nucleus dividing first. Coulter and Chamberlain (8) also call attention to the fact that after fertilization the egg seems to rest for a period, while free endosperm nuclei are being formed. While this may be true in a majority of cases in Ulmus (figs. 35, 36), many instances were found which seem to be at variance with it (fig. 37).

The formation of endosperm generally proceeds rapidly and takes place by free nuclear divisions, the nuclei being scattered through the cytoplasm of the sac. These nuclei, especially in the early stages, are enormous in size and multinucleolate (figs. 35, 36, 56), the nucleoli being so large as to be mistaken often for nuclei in the act of fusing, as mentioned by Strasburger (18) for Corydalis cava. The endosperm nuclei were often found to be in simultaneous division throughout the sac (fig. 50), and in no instance was a rudimentary cell-plate noted. As the development of endosperm progresses, the cytoplasm becomes more and more vacuolate, the nuclei take a parietal position and become smaller; yet throughout its existence the endosperm is characterized by large multinucleolate nuclei.

EMBRYO.

The first division of the fertilized egg is by a transverse wall (figs. 36-38). The terminal cell again divides, usually transversely (fig. 39), while the micropylar cell becomes vesicular although not

enlarging very much.

After a proembryo of three or sometimes four cells has been formed, the end cell is usually the first to divide by a vertical wall (fig. 40). However, many exceptions to this rule were found, fig. 42 showing the second as well as the terminal cell forming a vertical wall; while fig. 43 shows the second cell dividing first. Figs. 44 and 45 show anomalous forms of embryos in which no definite order of division can be discovered.

From the regular octant stage the development of the embryo is quite rapid and usually regular, the apical octants being the first to divide by periclinal walls, thus differentiating the dermatogen of the cotyledonary region (fig. 46). Almost immediately that of the hypocotyledonary region is formed in the same way (fig. 47). Fig. 48 shows early development of plerome, of periblem of the root, and the differentiation of the dermatogen of the root tip. A further study of the development of the embryo revealed nothing worthy of mention.

POLYEMBRYONY.

The discovery of pollen tubes near the chalazal region, as well as perfectly formed eggs in the antipodal end of the embryo sac, led me to suspect that antipodal embryos might be discovered associated with normally placed embryos. In several cases antipodal embryos were noted (figs. 51, 52), and in one case an extra-micropylar embryo (fig. 49). It is also likely that pseudo-polyembryony may result from the presence of two embryo sacs, as well-formed and probably fertilized eggs were observed in such cases (fig. 56).

SUMMARY.

1. The microsporangia are in the mother cell stage in the early part of February and probably pass the winter in this stage, forming tetrads at the first breaking of winter weather.

2. The tapetum is formed from the peripheral layer of sporoge-

nous tissue.

- 3. The pollen grains leave the tetrad stage March 16 to 18 and generally show tube and generative nuclei at this time. By March 23 the male cells appear, while the dehiscence of the sporangium occurs from March 25 to 27.
- 4. The single megaspore begins to germinate February 15, resulting in eight to sixteen and occasionally more free nuclei.
- 5. In many instances the pollen grains thrust tubes through the openings in the exine in from two to five directions before coming in contact with the stigma, but only the one gaining such contact develops.
- 6. The pollen tube generally enters through the micropyle, though it has been found piercing the nucellus at various places and even making its way down the funiculus; it may also branch profusely, but this seems to occur only in the cases of belated tubes.
- 7. The male cells leave the pollen grain as soon as the tube is 1^{mm} in length, remaining close to its tip, and were always found side by side; they appear in their early existence to be fastened together by their adjoining ends.
 - 8. The tube nucleus does not leave the pollen grain.
- 9. Double fertilization was observed, taking place March 28 to 31, the first male cell fusing with the endosperm nucleus.
- 10. The endosperm nucleus generally divides before the fertilized egg, forming large, multinucleolate nuclei.
- II. The embryo is of the massive type, the suspensor cell enlarging but little.
 - 12. An antipodal egg is not uncommon.
 - 13. Two embryos are occasionally found in the same sac.
- 14. Two embryo sacs are sometimes formed in a single nucellus with an egg apparatus in each.
- 15. Chalazogamy was not certainly found, but from indications it may occur.
- I am indebted to Professor John M. Coulter and to Dr. Charles J. Chamberlain for efficient direction and assistance.

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EXPLANATION OF PLATES VII-IX.

All figures were made with an Abbé camera lucida and reduced one-half in reproduction. Figures with a magnification greater than 600 diameters were made with a Zeiss apochromatic objective 2^{mm}, ap. 1.30, and Zeiss compensating oculars 4, 8, and 12. All others with Spencer 5^{mm} and 16^{mm} objectives and oculars 4 and 8.

The abbreviations employed in describing fhe figures are as follows: fl, flower; pt, pollen tube; po, polar nuclei; e, egg; en, endosperm nucleus; ed, endothecium; s, stigma; sy, synergids; smc, spore mother cells; f, fusion nucleus; at, antipodals; m, male cell; mm, megaspore; o, ovule; oi, outer integument; ii, inner integument; tn, tube nuclei; t, tapetum; gn, generative nucleus; cr, crown cells; n, nucellus; sh, stigmatic hair.

Fig. 1. Young flower showing stigma protruding and ready for fertilization.

Fig. 2. Flower cluster showing filaments and flower stalks in the center as first to elongate. ×5.

Fig. 3. Winter stages of microsporangium showing mother cells. ×600.

Fig. 4. Later stage showing the organization of tapetum from peripheral mother cells; other mother cells in synapsis. ×600.

Fig. 5. Section of microsporangium showing enlargement of endothecium, the breaking down of the tapetum, and the two inner layers of cells of the sporangium walls; also tetrads dissociating. ×600.

Fig. 6. Microspore mother cell in first mitosis. ×1260.

Fig. 7. Second mitosis of microspore mother cell. ×1260.

Fig. 8. Four-nucleate mother cell preparing to form tetrads. × 1060.

Fig. 9. Tetrads within the mother cell. X1000.

Fig. 10. Microspore showing tube and generative nuclei and openings in the exine. X1260.

Fig. 11. Portion of the exine showing holes through which pollen tubes emerge. X1200.

Fig. 12. Division of generative nucleus to form male cells. ×1260.

Fig. 13. Male cells attached by their adjoined ends; tube nucleus disintegrating. X1260.

Fig. 14. Male cells free and encircling disintegrating tube nucleus. X1260.

Fig. 15. Pollen tube as it usually appears. ×1260.

Fig. 16. Male nuclei in pollen tube. ×1260.

Fig. 17. Disintegrating microspore showing cyst formed at end of stigmatic hair. ×1260.

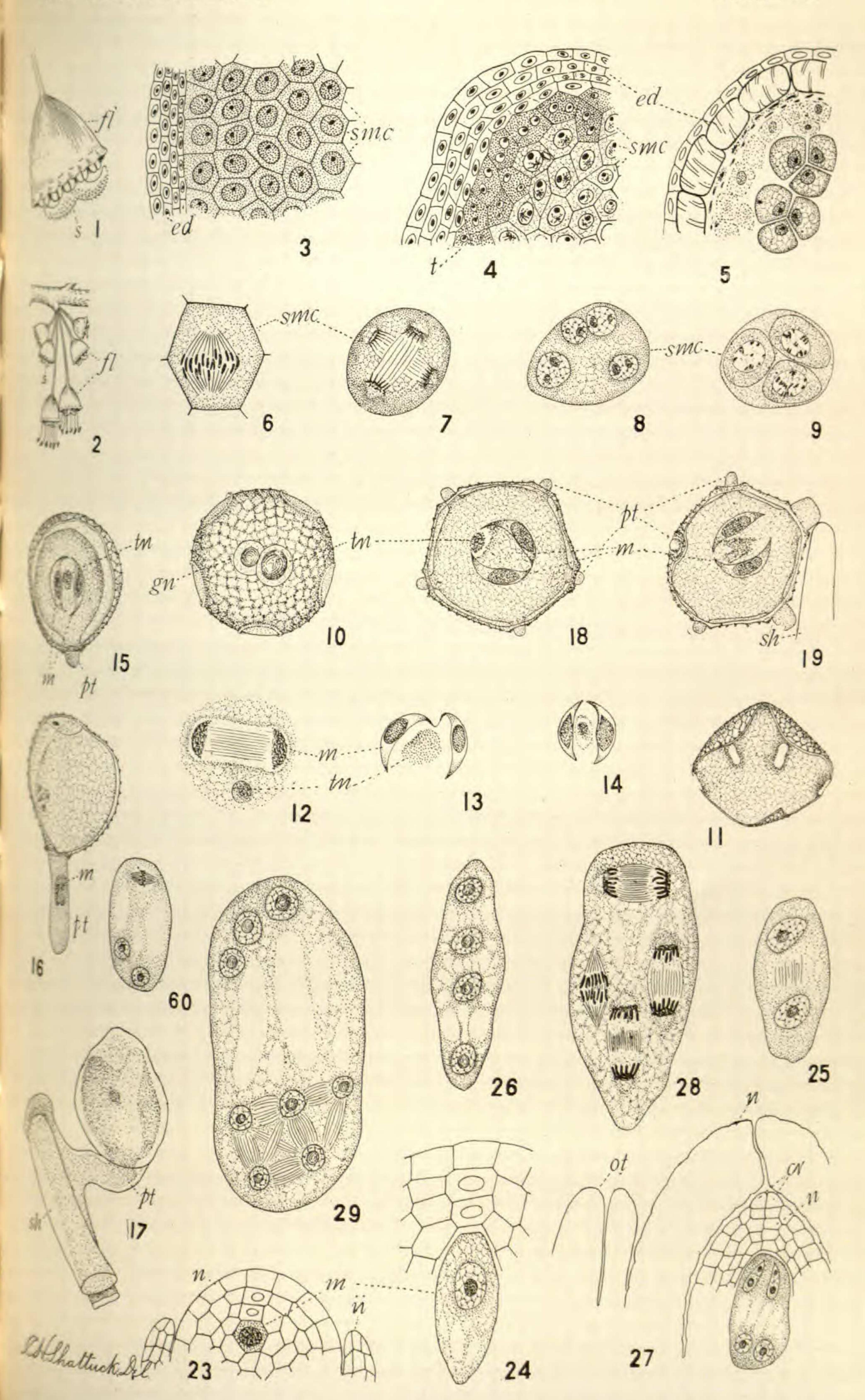
Fig. 18. Microspore showing five tubes emerging; all equal in size. X1260.

Fig. 19. Microspore showing five emerging tubes; one in contact with the stigmatic hair. ×1260.

Fig. 20. Two pollen tubes entering the same micropyle. ×450.

Fig. 21. Branching pollen tubes. × 200.

- Fig. 22. Anastomosing pollen tubes. × 200.
- Fig. 23. Megaspore mother cell showing nucellus and first integument. X1200.
 - Fig. 24. Later stage of megaspore. X1260.
 - Fig. 25. Binucleate stage of the embryo sac. X1260.
 - Fig. 26. Normal four-nucleate stage of embryo sac. × 700.
- Fig. 27. Frequent form of the four-nucleate stage of embryo sac; also a third integument. ×400.
 - Fig. 28. Mitosis of the four-nucleate sac. ×900.
 - Fig. 29. Eight-nucleate sac; unusual mitosis in antipodal end. X1200.
- Fig. 30. Usual eight-nucleate sac with the egg apparatus organizing and the polars approaching each others preparatory to fusion. ×900.
- Fig. 31. Embryo sac showing more than eight nuclei, but no sign of polarity. ×600.
 - Fig. 32. Multinucleate embryo sac showing polarity. ×900.
- Fig. 33. Eight-nucleate sac showing fusion of polars and entrance of pollen tube bearing the two male cells. ×1450.
- Fig. 34. Embryo sac showing one male cell in the act of fusing with the fusion nucleus, and the other near egg. ×1450.
- Fig. 35. First integuments; nucellus showing beak and bearing pollen tube; embryo sac showing first division of fertilized egg and multinucleolate nuclei of endosperm. ×500.
- Fig. 36. Embryo sac showing two-celled embryo unusually large; multi-nucleolate nuclei of rapidly forming endosperm; also an antipodal egg. ×810.
- Fig. 37. Embryo sac showing two-celled embryo, and the beginning of the formation of endosperm tissue. ×1200.
 - Fig. 38. Ordinary type of two-celled embryo. ×500.
 - Fig. 39. Three-celled embryo. ×500.
 - Fig. 40. Four-celled embryo showing first vertical wall. ×500.
- Fig. 41. Four-celled embryo showing usual method of formation of second vertical wall. ×500.
 - Fig. 42. Three-celled embryo; both end cells dividing at once. ×500.
- Fig. 43. Three-celled embryo showing second cell dividing before the end cell. ×500.
 - Fig. 44. Anomalous form of embryo. ×500.
 - Fig. 45. Anomalous form of embryo. ×500.
- Fig. 46. Later embryo showing the periblem in the cotyledonary region; also mitosis in the hypocotyledonary region. ×400.
 - Fig. 47. Embryo showing periblem in hypocotyledonary region. ×400.
- Fig. 48. Advanced embryo showing early development of periblem and dermatogen of root tip. ×400.
 - Fig. 49. Two embryos in the micropylar end of sac. ×500.
- Fig. 50. Well-formed embryo in micropylar end of sac; endosperm in simultaneous mitosis; egg-like formation in chalazal end. ×400.



SHATTUCK on ULMUS AMERICANA